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In Vitro Phosphorylation Study of the Arc Two-Component Signal Transduction System of *Escherichia coli*

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The ArcB and ArcA proteins constitute a two-component signal transduction system that plays a broad role in transcriptional regulation. Under anoxic or environmentally reducing conditions, the sensor kinase (ArcB) is stimulated to autophosphorylate at the expense of ATP and subsequently transphosphorylates the response regulator (ArcA). ArcB is a complex, membrane-bound protein comprising at least three cytoplasmic domains, an N-terminal transmitter domain with a conserved His292 residue (H1), a central receiver domain with a conserved Asp576 residue (D1), and a C-terminal alternative transmitter domain with a conserved His717 residue (H2). To study the phosphoryl transfer pathways of the Arc system, we prepared the following His-tagged proteins: H1, D1, H2, H1-D1, D1-H2, H1-D1-H2, and ArcA. Incubations of various combinations of Arc proteins with [γ-^{32}P]ATP indicated that H1, but not D1 or H2, catalyzes autophosphorylation; that H1-P transfers the phosphoryl group to D1 much more rapidly than to ArcA; and that D1 accelerates the transphosphorylation of H2. Finally, ArcA is phosphorylated much more rapidly by H2-P than by H1-P. Available data are consistent with a signal transduction model in which (i) reception of a membrane signal(s) triggers autophosphorylation of H1 at His292, (ii) the phosphoryl group can migrate to D1 at Asp576 and subsequently to H2 at His717, and (iii) ArcA receives the phosphoryl group from either His292 or His717, the relative contribution of which is regulated by cytosolic effectors.

The Arc two-component system is an important element in the complex transcriptional regulatory network that allows facultative anaerobic bacteria, such as *Escherichia coli*, to sense various respiratory growth conditions and adapt their gene expression accordingly (7, 14–16). The Arc system comprises the ArcB protein as the membrane sensor kinase and the ArcA protein as the response regulator. A redox signal associated with the plasma membrane is thought to stimulate the autophosphorylation of ArcB at the expense of ATP (11, 12). Phosphorylated ArcB catalyzes the transphosphorylation of ArcA, thereby activating it as a transcriptional regulator of a family of about 30 target operons (20), i.e., the Arc modulon (12). In this system, signal decay (dephosphorylation) appears to largely depend on the ArcB component. The half-life of phosphorylated ArcB is prolonged by fermentation products, such as D-lactate (10).

Paradigm sensor kinases consist of an N-terminal cytosolic segment, a membrane-spanning segment, a periplasmic domain, a second membrane-spanning segment, and a C-terminal cytosolic transmitter domain (25). However, ArcB belongs to a subclass of complex sensors with at least three cytosolic domains, an orthodox transmitter (H1 with a conserved His292), a medial receiver domain characteristic of response regulators (D1 with a conserved Asp576), and a C-terminal unorthodox transmitter domain (H2 with a conserved His717) (Fig. 1). Other examples of this subclass of sensor kinases include BarA, BvgS, EvgS, LemA, RteA, and TorS (2, 7, 22, 28, 29, 32). However, even among the members of this subclass of sensor kinases, ArcB is unusual in that it lacks a significant periplasmic domain (16).

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Previous in vitro studies of ArcB indicated that His292, Asp576, and His717 participate in phosphotransfer reactions (9, 10, 14, 30). In this study, we confirmed and extended these studies by analyzing the properties of individual ArcB transmitter and receiver domains and combinations thereof with the aim of further exploring the pathways for phosphoryl group transfer to ArcA. The results led us to propose a signal transfer model which differs significantly from those previously suggested (10, 13, 30).

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. *E. coli* M15 and plasmids pREP4 and pQE30 were obtained from Qiagen Ltd. The oligonucleotides listed in Table 1 were purchased from Oligos Etc. Plasmid pQE30ArcA, used for expression of a His-tagged variant of ArcA, has previously been described (19). In the construction of pQE30 derivatives that express similarly tagged portions of the ArcB protein (Table 1), Vent DNA polymerase (New England Biolabs) was employed in PCRs with pBB25 (13) as the template. All DNA fragments cloned from PCR-amplified material were sequenced to check that no undesired base changes had been introduced. DNA sequence analysis was performed by the HHMI/Harvard Medical School Biopolymers Facilities User Group.

Construction of vectors expressing ArcB subdomains. A series of derivatives of the pQE30 vector were constructed to facilitate simple purification of various fragments of the ArcB protein bearing an N-terminal oligohistidine (His_6) tag. The limits of the coding region of each subdomain, as defined by the sequences of the oligonucleotides used in PCR amplification (Table 1), were chosen on the basis of computer alignments of the ArcB sequence with those of other sensor kinases of the two-component system family according to the nonredundant peptide sequence database of September 1994. Sequence alignments were initially generated by using the BLASTP program run at the National Center for Biotechnology Information and were further refined by using programs of the Genetics Computer Group package (University of Wisconsin, Madison). The ArcB subdomains studied are listed in Table 1. For the purposes of this study, amino acid residues 1 to 77 (comprising the membrane-spanning segments of the protein) were omitted.

Purification of His_6-tagged proteins. *E. coli* M15 cells cotransformed with pREP4 and the appropriate pQE30 derivative were grown in 1 liter of medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl/liter) supplemented with 100 μg of ampicillin/ml and 25 μg of kanamycin/ml. The expression of six-His-tagged proteins was induced at mid-exponential phase (optical density at 600 nm of 0.7 to 0.8) by the addition of 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were harvested after 4 h of induction. Protein purification was performed at 4°C under nondenaturing conditions by a protocol (protocol 5)

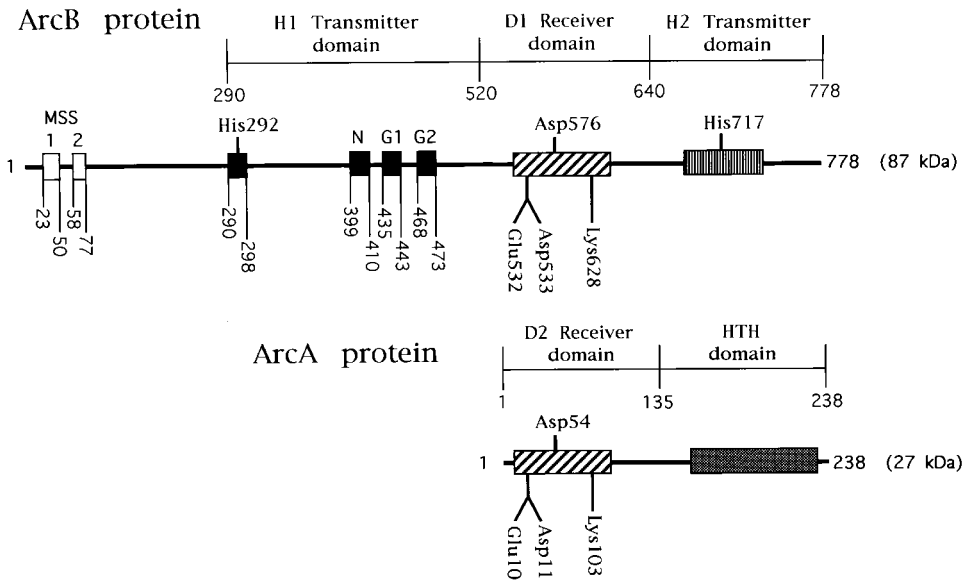

FIG. 1. Schematic representations of ArcB and ArcA. (Top) The two N-terminal membrane-spanning segments (MSS) of ArcB were predicted on the basis of a hydrophobicity plot (16). H1 (the orthodox transmitter domain) is shown with the conserved His292 (13) and the catalytic determinants N, G1, and G2 (24). G1 and G2 resemble nucleotide binding motifs. D1 (the receiver domain) is shown with the conserved Asp576 (13), and H2 (the secondary transmitter domain) is shown with the conserved His717 (9). (Bottom) ArcA is shown with its N-terminal receiver domain containing the conserved Asp54 and its C-terminal helix-turn-helix (HTH) domain (15).

previously published for native purification of cytoplasmic proteins (27a). Purification was based on affinity chromatography with the chelate absorbent Ni-nitrilotriacetic acid resin that interacts with the six-His tag. Proteins were eluted by imidazole, which was subsequently removed by dialysis (20). After dialysis, proteins were concentrated in Centricon 10 units (Amicon) and stored at −20°C. The Coomassie Plus protein assay reagent (Pierce) was employed to estimate protein concentrations, with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified proteins revealed that each had the expected molecular weight and that the preparations were essentially homogenous (see Fig. 2B). A His_6-tagged form of the CpxR protein, hereafter referred to simply as CpxR, was prepared as described previously (26). PhoB, a kind gift of M. Prahalad and C. T. Walsh (Harvard Medical School), was prepared as previously described (6).

Phosphorylation and transphosphorylation assays. Unless otherwise specified, phosphorylation assays were carried out at room temperature in the presence of 40 μM [γ-^{32}P]ATP (specific activity, 2 Ci/mmol; New England Nuclear)–33 mM HEPES (pH 7.5)–50 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol–0.1 mM EDTA–10% glycerol. The reactions were initiated by the addition of [γ-^{32}P]ATP, terminated by the addition of an equal volume of 4× SDS sample buffer, and immediately subjected to SDS-PAGE on 15% polyacrylamide gels (18). In time course experiments, samples were mixed with SDS sample buffer and kept on ice until the last portion was taken. After completion of electro-

TABLE 1. Derivatives of pQE30 constructed

Plasmid[a]	Primer	Sequence[b]	Theoretical molecular mass (Da)[c]
pQE30ArcB$^{78-778}$	Upstream	5′-CCCGGATCCCATATGgagcaactggaggagtcacgac-3′	80,917
	Downstream	5′-CCCGGATCCATGCAtcgcgcaccccggtctagcc-3′	
pQE30ArcB$^{78-661}$	Upstream	5′-CCCGGATCCCATATGgagcaactggaggagtcacgac-3′	67,823
	Downstream	5′-CCCGGATCCATGCATCATTAtgatttactgttctcttctgtcgtc-3′	
pQE30ArcB$^{78-520}$	Upstream	5′-CCCGGATCCCATATGgagcaactggaggagtcacgac-3′	52,234
	Downstream	5′-CCCGGATCCATGCATCATTAatcgtcttcatcaaacgcatcatcg-3′	
pQE30ArcB$^{521-778}$	Upstream	5′-CCCGGATCCCATatgcctttaccggcgctgaatgtgc-3′	30,235
	Downstream	5′-CCCGGATCCATGCAtcgcgcaccccggtctagcc-3′	
pQE30ArcB$^{521-661}$	Upstream	5′-CCCGGATCCCATatgcctttaccggcgctgaatgtgc-3′	17,141
	Downstream	5′-CCCGGATCCATGCATCATTAtgatttactgttctcttctgtcgtc-3′	
pQE30ArcB$^{638-778}$	Upstream	5′-CCCGGATCCCATATGatcaagaaattctgggataccc-3′	17,415
	Downstream	5′-CCCGGATCCATGCAtcgcgcaccccggtctagcc-3′	

[a] For each plasmid, the superscript numbers refer to the amino acid residues of ArcB which are included in the His-tagged protein encoded.

[b] PCR amplification products were generated with Vent DNA polymerase (New England Biolabs), digested with *Bam*HI (5′-G′GATCC-3′) and *Nsi*I (5′-ATGCA′T-3′), and the resulting products were cloned between the *Bam*HI and *Pst*I (5′-CTGCA′G-3′) of pQE30 (Qiagen Ltd.). The DNA sequence of the PCR-amplified region was subsequently verified for each plasmid. The underlined regions in the upstream and downstream primers correspond to the *Bam*HI and *Nsi*I sites, respectively; the lowercase sequences correspond to those of the *arcB* gene.

[c] The theoretical molecular mass of the His-tagged product encoded by each plasmid was calculated from the predicted sequence of the protein, which bears an amino-terminal Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser-His extension.

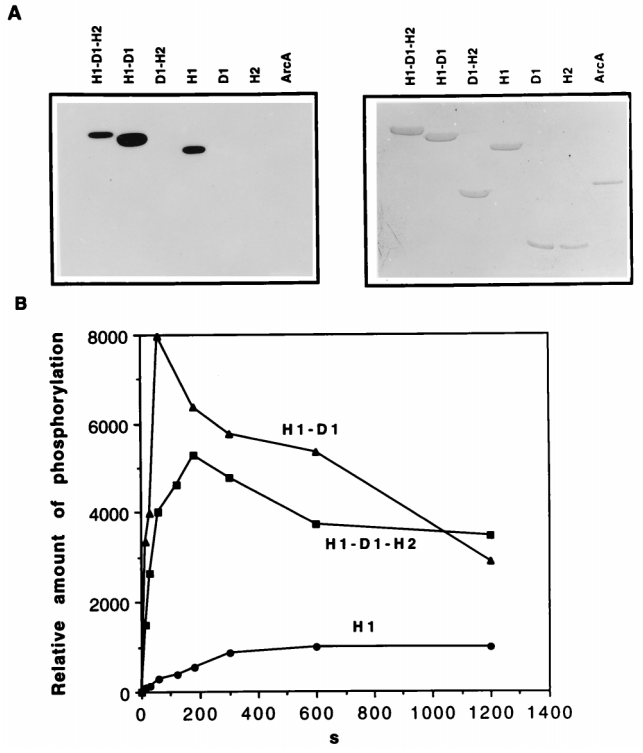

FIG. 2. Testing the autophosphorylation activities of ArcB fragments and ArcA. Purified proteins (~100 pmol [each]) were incubated with [γ-^{32}P]ATP for 2 min in a total volume of 5 μl, and the proteins in reaction mixtures were subsequently resolved by SDS-PAGE (see Materials and Methods). (A) Autoradiogram of the protein bands on the gel (left) and the same gel stained with Coomassie blue (right). (B) Time course (in seconds) of autophosphorylation of purified proteins. The reaction mixtures contained ~100 pmol of each purified protein in a total volume of 60 μl. At each indicated time point, a 5-μl sample was withdrawn for subsequent SDS-PAGE analysis and quantitation with a PhosphorImager.

phoresis, gels were stained by Coomassie blue R (Sigma) to reveal protein bands. The radioactivities of proteins resolved on gels were determined qualitatively by autoradiography of dried gels with X-Omat AR (Kodak). A PhosphorImager (Molecular Dynamics) was used for quantitative analyses.

RESULTS

Role of H1 in autophosphorylation. By amino acid sequence homology, His292 has been identified as the conserved site of autophosphorylation of the ArcB sensor kinase, and the conversion of His292 to a Gln residue has been found to relieve Arc-dependent anaerobic repression of a target reporter, Φ (*sdh-lac*) (13). To determine whether His292 of the H1 transmitter domain is the only site capable of autophosphorylation in ArcB, five His_6-tagged fragments of the protein, comprising different domains (Fig. 1), were incubated with [γ-^{32}P]ATP. Among the samples tested, $ArcB^{78\text{-}778}$ (H1-D1-H2), $ArcB^{78\text{-}661}$ (H1-D1), $ArcB^{521\text{-}778}$ (D1-H2), $ArcB^{78\text{-}520}$ (H1), $ArcB^{521\text{-}661}$ (D1), and $ArcB^{638\text{-}778}$ (H2), only those polypeptides that contained His292 underwent autophosphorylation. In a control reaction, the His_6-tagged ArcA protein (hereafter referred to simply as ArcA) failed to undergo autophosphorylation with ATP as the phosphoryl group donor (Fig. 2A), as expected from a previous report (14). It can thus be concluded that H1 is both necessary and sufficient for autophosphorylation and consequently must be the site for initiating the phosphoryl transfer cascade in the Arc system.

It may also be noted that the amounts of ^{32}P incorporated into H1-D1-H2, H1-D1, and H1 were not the same. A kinetic analysis showed that the initial rates of net autophosphorylation of H1-D1-H2 and H1-D1 were strikingly more rapid than that of H1 (Fig. 2B). However, the ^{32}P acquired by H1-D1-H2 and H1-D1 reached a peak and subsequently declined. In contrast, the labeling of H1 continued to increase during the course of the experiment for 5 min (Fig. 2B). These different patterns suggest that D1 has an associated phosphatase activity and/or readily loses the phosphoryl group from Asp576.

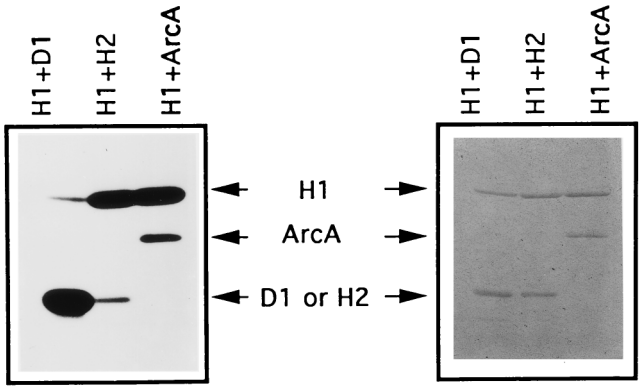

FIG. 3. Relative phospho-accepting activities of D1, H2, and ArcA from H1-P. H1 (100 pmol) was preincubated for 10 min with [γ-^{32}P]ATP in three separate 5-μl reaction mixtures, to which D1 (300 pmol), H2 (300 pmol), or ArcA (300 pmol) was added. The reactions were terminated 10 min later and analyzed by SDS-PAGE. (Left) Autoradiogram of the protein bands on the gel. (Right) Same gel stained with Coomassie blue. The positions of H1, D1, H2, and ArcA are indicated between the panels.

Preference of H1-P for D1 as a phosphoryl group acceptor. Since D1, H2, and ArcA did not autophosphorylate, their abilities to accept the phosphoryl group from H1-P were compared. Each of the three proteins was added to a reaction

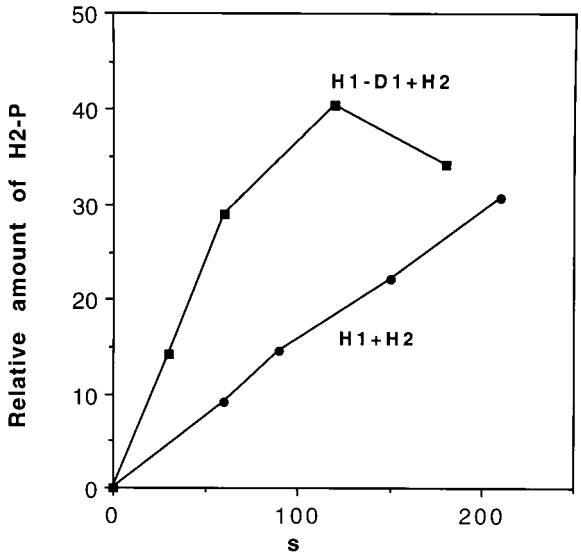

FIG. 4. H1-D1 is more effective than H1 is in catalyzing the net phosphorylation of H2 at the expense of ATP. H1 (100 pmol) was preincubated for 5 min with [γ-^{32}P]ATP in a 30-μl reaction mixture, followed by the addition of H2 (100 pmol). In a parallel reaction, H1-D1 (100 pmol) and H2 (100 pmol) were added simultaneously to the [γ-^{32}P]ATP-containing mixture (30 μl). Samples of 5 μl were withdrawn at the indicated time intervals. Time is shown on the *x* axis in seconds.

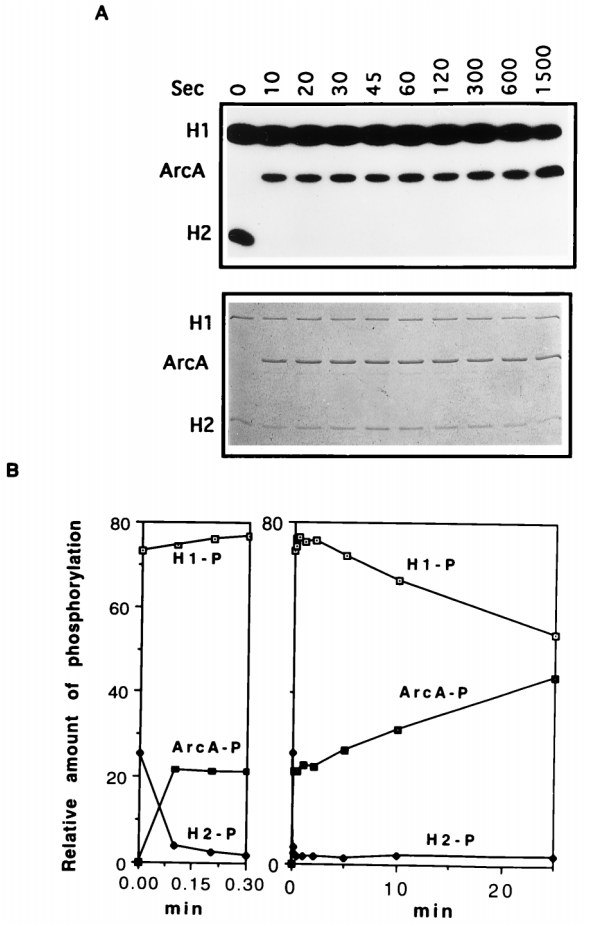

FIG. 5. ArcA phosphorylation by H1-P and H2-P. H1 and H2 (100 pmol of each) were preincubated with [γ-^{32}P]ATP for 45 min in a 60-μl reaction mixture to generate pools of H1-P and H2-P. At zero time, 3 μl of ArcA (100 pmol/μl) was added, and 5-μl samples were withdrawn at the indicated time intervals for analysis. (A) Autoradiogram of the dried gel (top) and the Coomassie blue-stained gel (bottom). (B) Kinetics during the initial 0.3-min period (left) and expanded plot of kinetics over a 25-min period (right).

mixture in which H1 was preincubated for 10 min with [γ-^{32}P]ATP. The results indicated that D1 was significantly more active than ArcA as an acceptor and that ArcA was in turn significantly more active than H2 (Fig. 3). The observation that H2 exhibited even a weak accepting activity should be noted, although this is apparently inconsistent with the requirement of complementarity between the reactive surfaces of phosphoryl donor and acceptor groups.

Effect of D1 on the phosphorylation of H2. To explore the influence of D1 on the phosphorylation of H2, two phosphoryl group donors were compared. In one experiment, H1 was incubated with [γ-^{32}P]ATP before the addition of H2 at zero time. In the other experiment, H1-D1 and H2 were added simultaneously to the [γ-^{32}P]ATP-containing mixture. Despite the autophosphorylation head start of H1, it catalyzed the phosphorylation of H2 at a rate slower than that of H1-D1 (Fig. 4). A likely explanation of this observation is that D1 accelerated the phosphorylation of H2 by effectively acting as both a phosphoryl group acceptor from H1-P and a phosphoryl group donor to H2.

Phosphotransfer from H2 to ArcA. The emerging possibility of a rapid phosphotransfer pathway from H1 to H2 via D1 raised the intriguing notion that H2-P is the most effective phosphodonor for ArcA. To test this hypothesis, H1 and H2 were incubated together with [γ-^{32}P]ATP for 45 min to form pools of H1-P and H2-P, ArcA was added, and the changes in the radioactivity associated with each protein were monitored (Fig. 5). ArcA rapidly gained the label, whereas H2-P rapidly lost the label. Moreover, during the initial minute, the amount of radioactivity gained by ArcA was approximately equal to that lost by H2-P. In contrast, during this initial period, no significant change in the radioactivity of H1-P was apparent. It may be noted that further increases in ArcA-P levels occurred very slowly and seemed to be at the expense of H1-P.

The importance of H2 in the rapid phosphotransfer pathway to ArcA is supported by the results from further studies. In one experiment, H1 and D1 were incubated with [γ-^{32}P]ATP for 2 min before ArcA was introduced. The rate of ArcA-P formation was monitored for 5 min, after which H2 was added. This addition rapidly accelerated the phosphorylation of ArcA (Fig.

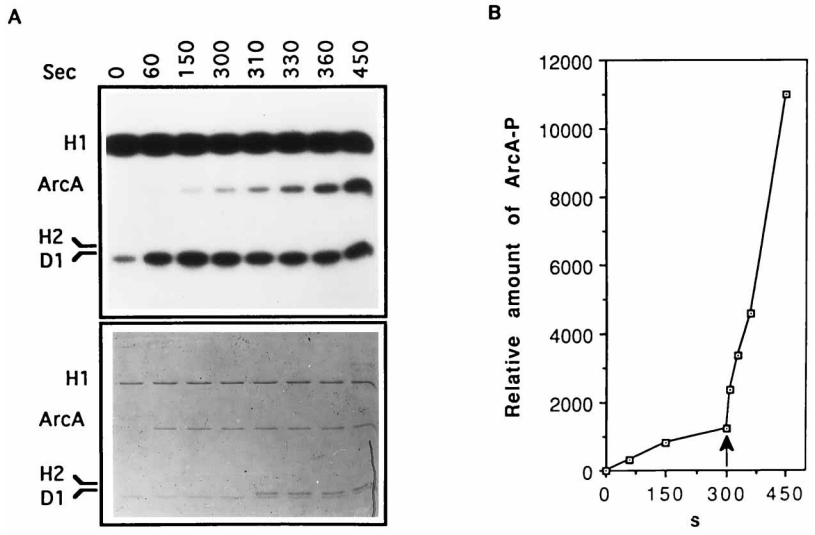

FIG. 6. Acceleration of ArcA phosphorylation by H2. H1 (50 pmol) and D1 (50 pmol) were preincubated with [γ-^{32}P]ATP in a 60-μl reaction mixture for 2 min. ArcA was added at zero time, and 300 s later, H2 was added. Samples of 5 μl were withdrawn at the indicated time intervals for analysis. (A) Autoradiogram of the dried gel (top) and the Coomassie blue-stained gel, revealing bands corresponding to H1, D1, ArcA, and H2 (bottom). (B) Relative amount of ArcA-P formed, as quantitated with a PhosphorImager. The arrow indicates the time of H2 addition to the reaction mixture.

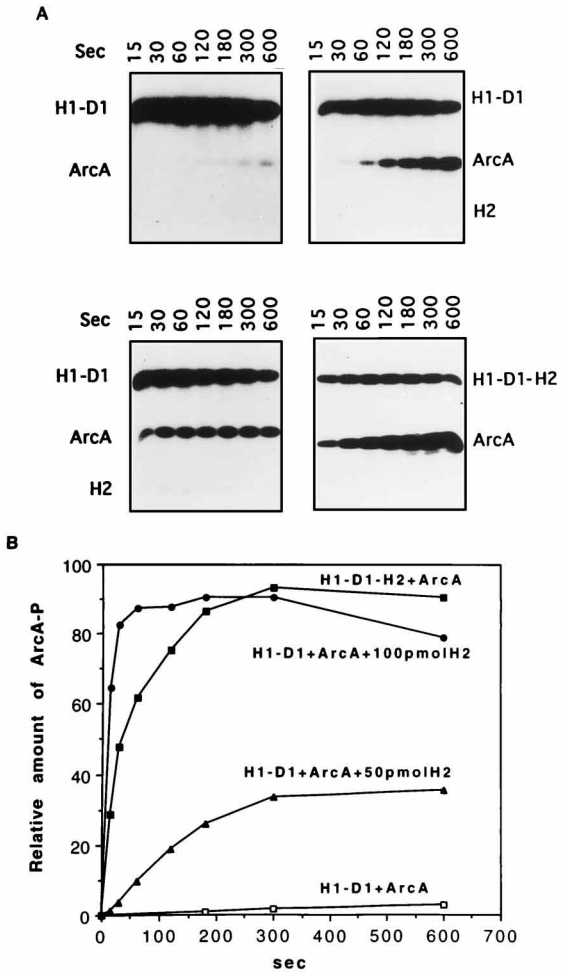

FIG. 7. Effect of H2 on the rate of ArcA-P formation. ArcA (50 pmol) was incubated with [γ-^{32}P]ATP and various ArcB fragments in a total volume of 30 μl. At the indicated time intervals, 3.5-μl samples were withdrawn. (A) Autoradiograms of dried gels. (Top) In the presence of H1-D1 (50 pmol) without (left) or with (right) H2 (50 pmol). (Bottom) In the presence of H1-D1 (50 pmol) with H2 (100 pmol) (left) and in the presence of H1-D1-H2 (50 pmol) (right). (B) Relative radioactivities acquired by ArcA under different incubation conditions, as quantitated with a PhosphorImager.

6). In another experiment, ArcA and [γ-^{32}P]ATP were incubated with different combinations of ArcB fragments and the kinetics of ArcA phosphorylation was monitored. H1-D1 barely stimulated the reaction. In contrast, rapid ArcA phosphorylation occurred in the presence of H1-D1-H2 or H1-D1 plus H2 and the effect of H2 was observed to be dependent on its concentration. For instance, 100 pmol of H2 plus 50 pmol of H1-D1 catalyzed the phosphorylation reaction of ArcA at a rate equal to or faster than that catalyzed by 50 pmol of H1-D1-H2 (Fig. 7). It is noteworthy that H2 acted in concert with H1-D1 without the necessity for a contiguous peptide linkage.

Test for H1 and H2 as phosphoryl group donors to cognate and noncognate response regulators. Whereas numerous examples of cross talk between sensor kinases and noncognate response regulators in vitro (1, 5, 8, 23) and in vivo (1, 5, 8, 17, 23) have previously been reported, evidence that such reactions are of physiological significance is lacking. However, in light of speculation that the carboxy-terminal H2 domain of ArcB is involved in cross signaling with other two-component systems (9, 30), we took advantage of H1-D1 and H2 to test the relative activities of the two ArcB transmitters as phosphoryl group donors to ArcA and the noncognate response regulators PhoB (21) and CpxR (4). As shown in Fig. 8, when the three response regulators were individually incubated with H1-D1 in the absence or presence of H2 for 2 min, significant phosphorylation occurred only with ArcA and only when H2 was present (Fig. 8). Thus, neither H1 nor H2 recognizes these noncognate response regulators.

DISCUSSION

The results from our phosphotransfer studies of different combinations of ArcB domains are qualitatively consistent with those from previous studies of ArcB^{c} (i.e., ArcB$^{Ile639\text{-}Lys778}$) and full-length wild-type and mutant ArcB proteins associated with everted membrane vesicles (30). The consistency of the results obtained by two experimental approaches validates our chosen partition sites for the ArcB domains and indicates that various H1-, D1-, and H2-containing polypeptides are able to fold into functional tertiary structures, with or without covalent linkage of the contiguous domains. Furthermore, N-terminal tagging

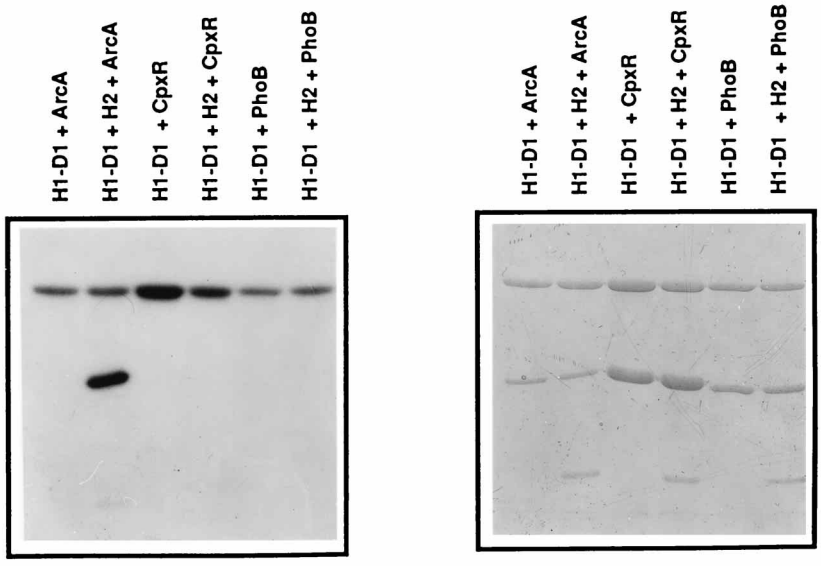

FIG. 8. Comparison of the abilities of H1-D1 to phosphorylate the ArcA, CpxR, and PhoB proteins and the influence of H2 on the reaction. H1-D1 (50 pmol) was incubated in 5-μl reaction mixtures with [γ-^{32}P]ATP for 2 min with ArcA (100 pmol), CpxR (200 pmol), or PhoB (100 pmol) in the presence (+) or absence of H2 (100 pmol). (Left) Autoradiogram of phosphorylated proteins on the dried gel. (Right) Same gel stained with Coomassie blue.

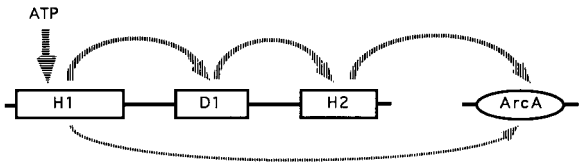

FIG. 9. Model for signal transduction by the Arc system, with special focus on the reactions leading to the phosphorylation of the response regulator, ArcA. Arrows indicate the directions of phosphoryl group transfer along two alternative signaling pathways, with the thicknesses of arrows indicating the relative activities of these pathways observed under the in vitro conditions of this study.

with a histidine oligopeptide did not seem to be functionally perturbing. It is thus feasible to characterize each domain separately. The success of this analytic method should facilitate meaningful transphosphorylation analyses in the future.

Several results from our biochemical studies deserve emphasis. First, H1 appeared to undergo autophosphorylation more rapidly when it was associated with D1 than when it was alone (Fig. 2). Second, the presence of D1 accelerated the phosphoryl group transfer from H1 to H2 (Fig. 4). Third, H2-P was a much more effective phosphoryl group donor to ArcA than H1-P (Fig. 5). Taken together, these observations suggest that signal transduction from ArcB to ArcA proceeds by two distinct pathways (Fig. 9).

Attention on the mechanism of action of ArcB has been diverted by a postulate that the role of the orthodox H1 domain is to transmit the signal to ArcA and that the role of the C-terminal H2 domain is to transmit or receive a signal(s) from a member of an unidentified two-component system(s). According to such a model, D1 mediates a reversible phosphoryl transfer reaction between H1 and H2, each of which possesses its own response regulator protein-substrate specificity. If this is true, intercommunication among different two-component systems may serve to coordinate various control circuits (9, 30). The cross-regulation model was based on the observation that replacement of His717 with a Leu residue in ArcB did not alter the normal expression pattern of the *lct* target operon, as measured by L-lactate dehydrogenase activity. Quantitative data and their reproducibility, however, were not presented (9). In contrast to the D1 adapter model, a previous series of in vivo experimental results, based on gradual deletions from the C-terminal end of ArcB, indicated that H1-D1 alone was insufficient to confer an $arcB^+$ phenotype, as judged by *lct* expression. In particular, $ArcB^{1-733}$ showed an essentially wild-type phenotype, but $ArcB^{1-668}$ showed a null phenotype (10a, 15).

In view of the in vivo results cited above and the in vitro results described here, it seems that another model for ArcB signaling (Fig. 9) should be considered and tested. This alternative model involves ArcA receiving the phosphoryl group from either the H1 or H2 pathway, with the relative contribution of each determined by the cytosolic environment of ArcB. Specifically, cytosolic effectors, such as D-lactate, may play a decisive role in this regard by inhibiting the dephosphorylation of ArcB (10).

The notion of a direct phosphoryl group transfer from the C-terminal transmitter of an unorthodox sensor kinase to its cognate response regulator is not novel. Indeed, phenotypic characterization of mutant *Bordetella pertussis* BvgS, another sensor kinase of the ArcB subfamily, indicated that the conserved C-terminal His serves as the obligatory phosphoryl group donor for the response regulator BvgA (31). A multistep phosphorelay, His→Asp→His→Asp, was reported for the Kin/Spo system of *Bacillus subtilis* (3) and the Sln1p/Ypd1p/Ssk1p of *Saccharomyces cerevisiae* (27). Knowledge of the location and mechanism(s) of dephosphorylation of the Arc components should cast further light on the modus operandi of signal transduction in this system.

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